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# Low temperature during infection limits *Ustilago bullata* (Ustilaginaceae, Ustilaginales) disease incidence on *Bromus tectorum* (Poaceae, Cyperales)

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## Abstract

*Ustilago bullata* is frequently encountered on the exotic winter annual grass *Bromus tectorum* in western North America. To evaluate the biocontrol potential of this seedling-infecting pathogen, we examined the effect of temperature on the infection process. Teliospore germination rate increased linearly with temperature from 2.5 to 25°C, with significant among-population differences. It generally matched or exceeded host seed germination rate over the range 10–25°C, but lagged behind at lower temperatures. Inoculation trials demonstrated that the pathogen can achieve high disease incidence when temperatures during infection range 20–30°C. Disease incidence was drastically reduced at 2.5°C. Pathogen populations differed in their ability to infect at different temperatures, but none could infect in the cold. This may limit the use of this organism for biocontrol of *B. tectorum* to habitats with reliable autumn seedling emergence, because cold temperatures are likely to limit infection of later-emerging seedling cohorts.

**Keywords:** Bromus tectorum, *cheatgrass, downy brome, head smut, infection window,* Ustilago bullata, *weed biocontrol*

## Introduction

*Bromus tectorum* L. (downy brome, cheatgrass; Poaceae, Cyperales) is a serious and difficult-to-control weed of winter cereal grains in western North America (Peeper 1984) and is even more important as a weed of wildlands in this region (D'Antonio & Vitousek 1992). Following its introduction in the late 1800s, probably as a contaminant in grain seed, it rapidly increased in distribution and abundance in the wake of abusive livestock grazing (Mack 1981). The invasion of semiarid ecosystems of the Interior West by *B. tectorum* has been called the most significant plant invasion in the history of North America (D'Antonio & Vitousek 1992). Because this winter annual grass forms a continuous fine fuel that dries early in the summer, its dominance is associated with a dramatic increase in the frequency and size of wildfires (Whisenant 1990). Because *B. tectorum* responds positively to the

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disturbance created by fire, the result over time is dominance over ever-increasing areas.

While there is little hope of *B. tectorum* eradication in this region, its deleterious effects can be ameliorated on a local scale through restoration of native shrub-grass communities. Unfortunately, direct seeding into sites dominated by *B. tectorum* is rarely successful (Monsen 1994). In winter-rainfall climates where this weed is a problem, perennial seedlings on *B. tectorum*-dominated sites typically show very low over-summer survival because of soil moisture depletion by this winter annual weed. In order to restore these plant communities, some form of *B. tectorum* control is necessary. Presently available alternatives include scalping, tillage after fall emergence, early season burning (while seed is still on the plants), and herbicide use. These methods have disadvantages, including excessive cost, risk, and damage to remnant perennials. A biocontrol agent that could target *B. tectorum* specifically would greatly increase the feasibility of restoration through direct seeding in these ecosystems. Our research focuses on the possibility of using an indigenous fungal pathogen, *Ustilago bullata* Berk. (Ustilaginaceae, Ustilaginales), as a *B. tectorum* biocontrol organism (Meyer et al. 2001).

*Ustilago bullata,* the causal organism for head smut disease of grasses, has a wide host range and a worldwide distribution (Fischer & Holton 1957). It is very commonly encountered on *B. tectorum* in western North America, sometimes reaching epidemic proportions (Fischer 1940; Fleming et al. 1942; Stewart & Hull 1949; Mack & Pyke 1984; Gossen & Turnbull 1995). Even though the pathogen species as a whole has a wide host range, host species, and even populations or genotypes within species, often support genetically distinct pathotypes of the fungus (Fischer 1940). Multiple pathotypes occur on *B. tectorum* populations in western North America, though most are pathogenic on a majority of *B. tectorum* host lines (Meyer et al. 2005). In extensive investigations on the host-specificity of *U. bullata* pathotypes, we almost never found pathotypes from *B. tectorum* that could infect native grass species (Meyer, unpublished data). This strongly suggests that *U. bullata* races on *B. tectorum* originated in the Old World range of the host and traveled with it to the introduced range.

*Ustilago bullata* is a seedling-infecting pathogen that grows systemically in the heterokaryotic phase, then sporulates in the host influorescence, producing masses of diploid teliospores (Fischer & Holton 1957). These teliospores disperse, then germinate and undergo meiosis to produce four haploid cells. Normally each of these cells is capable of either fusion with a cell of opposite mating type or saprophytic mitotic proliferation to produce haploid sporidia. The sporidial phase functions to increase infection probability by multiplying the number of cells capable of forming infective hyphae. Fusion of cells of opposite mating type is necessary for the production of the dikaryotic infection hypha that penetrates the seedling and initiates systemic mycelial growth. Sporulation by the pathogen almost always eliminates seed production. This effectively terminates the life cycle of an annual grass host.

Falloon (1979a) examined the infection process in detail for *U. bullata* on the perennial forage grass *Bromus catharticus*. He determined that the site of infection was the immature coleoptile, and that the window of infection was during the time after coleoptile emergence but prior to coleoptile elongation to 2 cm. He also found that, over the range of temperatures from 12 to 25°C, the development of the pathogen was closely attuned to seedling development of its host (Falloon 1979b).

Turnbull and Gossen (2000) found that different pathotypes of *U. bullata* from native and introduced host grasses on the Canadian prairies had contrasting responses to both temperature and salinity. The pathotype from *B. tectorum* had teliospores whose germination was more suppressed at high temperature and salinity than those of the pathotype from the early summer-germinating halophyte *Hordeum jubatum* L. so that host and pathogen had apparently matching germination requirements. Fischer (1937), on the other hand, reported no differences in teliospore germination among 19 western North American *U. bullata* collections from a range of hosts. Germination response at temperatures below 15°C was not addressed in either study.

Falloon (1979a) reported that *U. bullata* sporidial proliferation on solid medium increased with temperature over the range 12–20°C, i.e. doubling time decreased, while Meiners (1947) reported that sporidial cultures from different pathogen races had temperature optima from 20 to 25°C but differed in growth at super and sub-optimal temperature. Turnbull and Gossen (2000) found no difference among pathotypes in sporidial doubling time at 20°C.

Effective infection by *U. bullata* requires close synchronization with host seed germination. *Bromus tectorum* seeds can germinate at any time from early autumn through mid-spring, depending on environmental conditions (Mack & Pyke 1983). The seeds are dormant at dispersal in early summer, but lose dormancy under dry summer conditions and are able to germinate quickly over a wide temperature range in response to autumn rains (Meyer et al. 1997; Bauer et al. 1998). If the autumn is dry, or if the microenvironment for a particular seed is dry, winter or spring germination is also possible. The seeds can germinate at near-freezing temperatures, but their germination in the cold is delayed (Meyer et al. 1997). The plants require vernalization in order to flower, but both imbibed seeds and seedlings can be vernalized, so that all individuals flower synchronously in late spring, regardless of their emergence time (Meyer et al. 2004).

In this study we focused on temperature, an important environmental factor that mediates host–pathogen interactions during the infection process. Our investigation examined variation in *U. bullata* populations on *B. tectorum* from a wide range of environments in five states (Table I). We sought to determine: (1) how temperature immediately before infection affects teliospore germination and sporidial proliferation, as well as host seed germination and seedling growth; (2) how rates of host and pathogen development interact at different temperatures to influence disease incidence levels; and (3) whether there are different ecotypes of the pathogen that have contrasting optima or different degrees of tolerance for super- or suboptimal conditions during development and infection. Our goal was to understand how environmental factors might limit disease incidence in the field, and to seek ways to circumvent these limits, possibly through selection of a pathogen ecotype with wider environmental tolerances.

## Materials and methods

### Teliospore collection and storage

*Ustilago bullata* teliospores were collected from eight pathogen populations selected to represent different habitats, elevations, and climatic zones, from warm and cold deserts to the foothills and mountains in the Intermountain West, USA (Table I). At least 20 randomly selected smutted host plant influorescences from each population

Table I. Site information for *Ustilago bullata* populations where teliospore collections and seed collections from co-occurring host populations were obtained.

| Site | State | Latitude longitude | Elevation (m) | Vegetation type | Annual precipitation (mm) | Mean January temperature (°C) | Mean July temperature (°C) |
|------|-------|--------------------|---------------|-----------------|---------------------------|-------------------------------|----------------------------|
| Arrowrock | Idaho | N43°32.8′ W115°47.9′ | 1150 | Montane– ponderosa pine | 600 | −4.4 | 19.1 |
| Buckskin Canyon | Nevada | N41°45.5′ W117°32.0′ | 2130 | Canyon riparian | 500 | −2.7 | 18.3 |
| Hobble Creek | Utah | N40°9.7′ W111°30.5′ | 1530 | Foothill sagebrush– gambel oak | 400 | −2.1 | 24.8 |
| Moses Lake | Washington | N47°17.5′ W119°13.9′ | 390 | Cold desert sagebrush | 200 | −3.4 | 21.7 |
| Potosi Pass | Nevada | N35°60.0′ W115°28.7′ | 1500 | Warm desert blackbrush– juniper | 250 | 1.7 | 26.5 |
| Sagehen Hill | Oregon | N43°32.0′ W119°17.2′ | 1400 | Sagebrush steppe | 280 | −3.3 | 19.3 |
| Strawberry | Utah | N40°14.7′ W111°9.1′ | 2400 | Montane meadow | 560 | −7.8 | 16.1 |
| Whiterocks | Utah | N40°17.3′ W112°49.7′ | 1560 | Cold desert– shadscale | 180 | −2.3 | 25.8 |

were placed in a sealed paper bag, and later screened to release spores. To collect teliospores, sori were ruptured by rubbing on a 40-mesh soil sieve; spores were then passed through a series of sieves ending with a 20-μ sieve. Preliminary testing indicated that teliospores exhibited varying degrees of dormancy at harvest and that this dormancy disappeared during dry storage (Meyer unpublished). Teliospore collections were stored in sealed vials at room temperature for at least 16 weeks post-harvest to ensure that they were non-dormant prior to initiation of germination experiments.

*Teliospore germination*

Spores of each of the eight teliospore collections were suspended in 1% sterile Tween 80 and sprayed using a vaporizer onto microscope slides coated with a thin layer of potato dextrose agar (PDA). The slides were set on U-shaped glass rods laid on wet filter papers inside sterile glass Petri dishes. The Petri dishes were placed in growth chambers at 2.5, 5, 10, 15, 20, and 25°C. Based on preliminary results, the slides were read at different time intervals from 4 to 984 h depending on temperature, with at least four read times per temperature. There were two slides per read time for each temperature × teliospore population combination. Ten randomly selected fields of view on each slide were examined with a compound microscope at ×200 magnification, and germination proportion was estimated by counting germinated and ungerminated teliospores in each field of view. Visible protrusion of the promycelium (or dikaryon) was the criterion for germination. For each of two slides for each treatment combination, the proportions of spores germinating after a given time

interval in ten fields of view were averaged and treated as a single replicate. These proportions were then expressed in terms of total viable teliospores by dividing by the proportion of viable spores. Germination proportion after 48 h at 25°C was used as the estimate of maximum viability; this value varied from 0.80 to 0.95 depending on teliospore population.

To compare results from temperatures that resulted in widely differing time courses for teliospore germination, we used mean germination rate (the inverse of mean germination time, i.e. hours to 50% germination of total viable teliospores$^{-1}$) as the response variable. This variable is routinely used in analyses of seed germination because it generally increases linearly with temperature (e.g. Bauer et al. 1998). Mean germination time values were derived by linear interpolation from the germination time courses for each treatment replication. These were expressed as rates and subjected to statistical analysis.

We used analysis of covariance (ANCOVA) to analyze the teliospore germination data set. ANCOVA can be used to compare slopes and intercepts from linear regression for multiple levels of a class variable in response to a continuous variable (Zar 1984). In this design, pathogen population was the fixed class variable and temperature was the continuous variable.

Because of very slow, erratic, and abnormal germination of teliospores at 2.5°C, germination response at this temperature was excluded from the ANCOVA. These data were analyzed with analysis of variance (ANOVA), with pathogen population as the independent variable and germination proportion at 984 h as the response variable. Data were expressed as proportion of viable spores and arcsine square root-transformed to improve homogeneity of variance prior to analysis.

*Seed germination and the infection window*

Host florets (hereafter referred to as seeds) were collected from co-occurring *B. tectorum* populations corresponding to each of the eight pathogen populations (Table I). Seeds for each of the four southern populations (Potosi Pass, Hobble Creek, Strawberry, and Whiterocks) were represented by greenhouse-grown progeny of four randomly selected parental lines, while the four northern populations were represented by field-collected seeds of four randomly selected parental lines. Seeds were stored at room temperature until fully non-dormant (at least 6 months) prior to initiation of experiments. Germination was assessed by incubating the seeds at 10, 15, 20, and 25°C. Ten seeds were placed on wet filter papers in Petri dishes and placed in incubators. The dishes were replicated five times for each treatment combination and read at 24, 48, 72, and 96 h. Radicle emergence to 1 mm was the germination criterion.

We used ANCOVA for a completely randomized design to examine the germination response of seeds from the eight host populations, with population as the fixed class variable and temperature as the continuous variable. Mean germination time was derived by linear interpolation on seed germination time courses as described previously, and mean germination rate (hours to 50% germination of viable seeds$^{-1}$) was used as the response variable.

To quantify more precisely the time-temperature relations for seedling parameters that influence the period of susceptibility for the host, we performed a germination experiment that included seeds of two host lines also used in growth chamber

inoculation trials (see below). For each of the two host lines at each temperature, 10 replications of 10 seeds were set on wet filter papers in Petri dishes and placed in incubators. The seeds were incubated at 2.5, 5, 10, 15, 20, and 25°C. Germination and seedling growth time course data were taken by recording for each dish the number of seeds germinated, the number of seeds with coleoptiles emerged, and the number of seeds with coleoptiles elongated to 2 cm, at five time intervals that varied depending on temperature.

Linear interpolation was used to estimate time to 50% germination, time to 50% coleoptile emergence, and time to 50% coleoptile elongation to 2 cm for each replicate of each line × temperature combination. Rate (i.e. hours to $50\%^{-1}$) was used as the response variable in ANCOVA, with host line as the fixed class variable and temperature as the continuous variable. ANCOVA was performed for each of the three seedling growth response variables.

## Sporidial proliferation

The effect of temperature on sporidial proliferation for isolates from eight pathogen populations was determined using optical density as a population measure. The isolates were grown in potato dextrose broth (PDB) culture in 50-mL flasks in shakers placed in growth chambers set at 2.5, 15, and 25°C. A stock suspension was prepared at room temperature for each of the 40 sporidial isolates. Each stock suspension was prepared from a single monosporidial culture. The stock cultures were produced according to the protocol in Meyer et al. (2001) and stored in 5-mL tubes at −80°C prior to the experiment. Sporidial stock cultures of 100 µl were pipetted into 50-mL flasks containing 20 mL of PDB. Five isolates were included for each of the eight populations. Three replications were included for each sporidial isolate × temperature combination (except in a few cases where individual replicates were lost to contamination). Because of problems with contamination, a separate flask was used for each of four read times, for a total of approximately 1500 flasks. Read times at each temperature were determined from preliminary data and took place during the exponential growth phase. For optical density determination, the contents of the flask were centrifuged using a Damon IEC HN-S centrifuge for 10 min at 2000 rpm, the supernatant discarded, and the pellet resuspended in distilled water. The pellet and the distilled water were mixed using a Thermolyne 37600 mixer and then read through a Bausch and Lomb Spectronic 700 set at 600 nm. The sporidial population was calculated based on a calibration curve developed using hemacytometer counts of sporidial cultures over a range of optical densities. The values were corrected for differences in initial population density prior to analysis.

To analyze the sporidial proliferation data, we used ANCOVA for a partly nested design, with population as a fixed class effect, temperature as a continuous effect, and isolate as a random effect nested within population. The experiment was not analyzed as a block design because, even though the replicates for each isolate × temperature combination were run at different times, a run did not include all possible combinations due to space constraints. We therefore treated the experiment as completely randomized rather than blocked. The response variable for ANCOVA was mean proliferation rate (hours to $3 \times 10^7$ sporidial cells-mL$^{-1}$). This population density represents approximately half the maximum density that was achieved under our cultural conditions, so that mean proliferation time is analogous to mean

germination time for teliospores. Preliminary analysis showed that a log-transformation of mean proliferation rate resulted in a variable that increased linearly with temperature; we therefore used log (mean proliferation rate) as the response variable in our analysis.

To examine differences in response to temperature among isolates within populations, we used Levy's test (Zar 1984) for significant differences among variances. For each population, we compared variances at the three temperatures. These variances include error (among replication) variance as well as among-isolate variance, but error variance is assumed to be uniform across populations and treatments, so any significant differences in population variance are considered due to differences in among-isolate variance.

*Growth chamber inoculation trials*

We conducted a growth chamber experiment to test the effect of pathogen population, temperature, and inoculum density on disease incidence. The experiment was carried out twice. Each of the two experimental trials had a randomized block design with eight populations, six temperatures (2.5, 5, 10, 15, 20, and 25°C), two inoculum densities and two blocks for a total of 192 experimental units. An experimental unit consisted of a group of 18 seeds that were inoculated and planted into individual cells. The two trials were identical except for the use of different Whiterocks host lines (Table I). This change was necessitated by inadequate seed supplies of any one host line at the initiation of the experiment. Both host lines were known to be highly susceptible to all eight pathogen populations (Meyer et al. 2001, 2005).

Seeds were inoculated with dry teliospores at two densities (3.7 mg and 0.3 mg/g seeds) by sealing 36 de-awned seeds in a small vial with the correct quantity of teliospores and applying mechanical vibration. These inoculum levels correspond to approximately 5000 and 400 teliospores per seed, respectively. These levels probably greatly exceed natural inoculum loads except possibly during severe epidemics. They were chosen on the basis of preliminary trials to maximize infection but still obtain a response to variation in inoculum level. The seeds were planted in 72-cell root trainer trays (Spencer Lemaire, Edmonton, Alberta, Canada) containing a planting mixture composed of four parts vermiculite, three parts peat moss, two parts field soil (a sandy loam from Point of the Mountain, Utah), two parts sand, and one part calcined montmorillonite clay. The mixture was steam-pasteurized for 90 minutes at 60°C prior to filling.

The inoculated seeds were planted in individual cells and the planted trays were incubated at each temperature in growth chambers in the dark. We kept the trays in each controlled-temperature environment until emergence was at least 90% complete. Seedlings were considered emerged when the plumule was above the tip of the coleoptile. After emergence, the plants were transferred to the greenhouse where they were allowed to grow for 3 weeks or until the appearance of the fourth true leaf. At that stage, they were moved to a cold room with an average temperature of 2.5°C and 8-h days for vernalization (Meyer et al. 2004). After 10–12 weeks in the cold room, they were then brought back to the greenhouse and allowed to grow to flowering. Disease incidence was assessed by determining the

proportion of smutted plants as evidenced by inflorescences filled with sori containing teliospores of the pathogen.

Statistical analysis of the growth chamber inoculation experiment used the SAS mixed model procedure for a split plot design, with time repetition and temperature as main plot factors, block nested within time repetition and temperature, and pathogen population and inoculum density as subplot factors for data analysis (SAS Version 8, MIXED Procedure; Quinn & Keough 2002). Population, temperature, and inoculum density were treated as fixed main effects and block and time repetition as random effects. A preliminary examination of the shape of the temperature response showed that the response was not linear; we therefore treated temperature as a class effect. Disease incidence proportion was arcsine square root transformed to improve homogeneity of variance prior to analysis. The Satterthwaite approximation option was used to set denominator (error) degrees of freedom for each effect and interaction.

*Field inoculation trial*

A field inoculation trial was conducted to determine whether epidemic levels of disease could be induced under field conditions through artificial inoculation of seeds, and to evaluate the effect of pathogen population, inoculum density, and planting date (a surrogate for temperature during emergence) on disease incidence. The trial was conducted at the Brigham Young University Spanish Fork Farm using field-collected seeds of at least 20 randomly selected individuals from the White-rocks *B. tectorum* population (Table I). We employed a randomized block design with 10 blocks. Pathogen population (eight populations), planting date (two dates), and inoculum density (two levels) were fixed main effects, for a total of 320 experimental units. Each experimental unit was represented by a 0.093-m$^2$ plot. The seeds were inoculated at two densities (3.7 and 7.4 mg/g) using the protocol described for the growth chamber experiment and broadcast sown at the rate of 50 viable seeds per plot. Planting dates were 5 October (early planting) and 7 December (late planting) 2001. Because the seeds used in the experiment were field-collected and could have been contaminated with teliospores, we included an uninoculated control plot in each block. These plots were seeded on the early planting date. Mean disease incidence in the control treatment was <1%; data from this treatment were not analyzed further. Each plot was mulched with autoclaved dried *B. tectorum* litter (20 g per plot) after planting. No supplemental irrigation was applied. Seedling emergence was evident by early November in the mid-autumn planting, while seedlings in the early winter planting did not emerge until shortly after snowmelt the following March. Disease incidence was assessed in June 2002 by counting healthy and smutted plants in each plot.

The field experiment was analyzed using mixed model analysis of variance (Quinn & Keough 2002). The response variable (disease incidence) was arcsine square root transformed prior to analysis to improve homogeneity of variance. The Satterthwaite approximation option was used to set denominator (error) degrees of freedom for each effect and interaction.

## Results

### Teliospore germination

Teliospore mean germination rate (hours to 50% germination of viable teliospores$^{-1}$) averaged over eight pathogen populations increased rapidly as a linear function of temperature over the range from 5 to 25°C (df = 1, 64; $F$ = 1176.36; $P$ < 0.0001; Figure 1). The mean rate at 5°C corresponded to a mean germination time of 154 h, while the rate at 25°C corresponded to a mean germination time of only 8 h.

The temperature × population interaction for teliospore mean germination rate was also highly significant (df = 7, 64; $F$ = 4.11; $P$ = 0.0009), indicating that teliospores from different populations differed in their germination response to temperature (i.e. slopes were significantly different among populations, Figure 1). Mean germination rate for collections from Whiterocks and Moses Lake increased slowly as temperature increased. Mean germination rate for collections from Arrowrock and Sagehen Hill showed steep increases with temperature, while the remaining collections were intermediate in their response.

The regression equation derived from the analysis of covariance temperature main effect (i.e. the solid line in each panel of Figure 1) predicted that teliospore germination rate would reach a value of zero at 4.9°C (germination rate = 0.00611(temperature) − 0.029). In fact, teliospores were able to germinate at 2.5°C, albeit very slowly. There were significant differences among populations in teliospore germination percentage after 6 weeks at 2.5°C, with percentages ranging from 12 to 65% (df = 7, 152; $F$ = 50.50, $P$ < 0.0001). Only one of the eight teliospore collections exceeded 50% germination. An approximate estimate of mean germination rate overall at 2.5°C was obtained by linear extrapolation from initial germination percentage (0%) and mean 984-h germination percentage (30%). This estimated rate was 0.00057 h$^{-1}$, which corresponds to a mean germination time of 1756 h or 10.3 weeks, an order of magnitude longer than the 154 h required at 5°C.

Teliospore germination at 2.5°C also differed qualitatively from germination at higher temperatures (Figure 2). At 25°C, the teliospores produced a metabasidium that subsequently budded off numerous sporidia, as has been reported previously for this species (Falloon 1979c). Dikaryon formation usually involved sporidial mating at this temperature, although cells of the metabasidium were sometimes involved. But at 2.5°C, the teliospores almost always germinated directly to the dikaryotic stage, with meiosis and mating apparently taking place inside the spore wall. Usually two dikaryons emerged from the spore, suggesting that mating took place between the primary products of meiosis. This severely restricted the production of sporidia and would greatly decrease the number of infective dikaryotic hyphae that could be formed.

### Seed germination and the infection window

More important for a seedling-infecting pathogen than the absolute teliospore germination rate at a given temperature is the relationship between teliospore germination rate and seed germination and seedling development rate. In the experiment examining seedling growth parameters as a function of temperature for two host lines, differences between the two lines were not significant, but there was a highly significant positive linear relationship between mean rate and temperature for
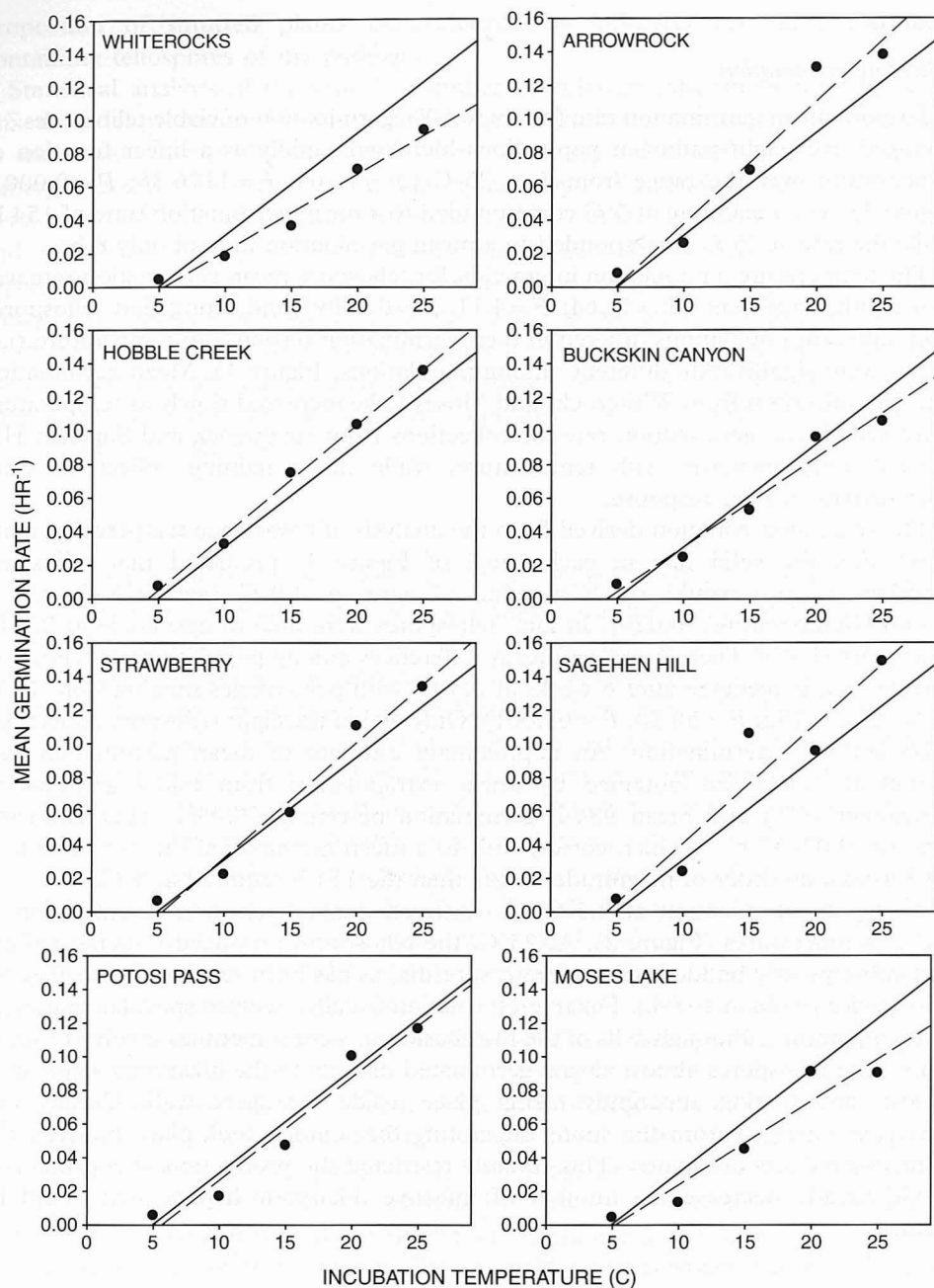
Figure 1. The relationship between mean germination rate and temperature for teliospore collections from eight *Ustilago bullata* populations. The dotted line in each panel is the population regression line, while the solid line is the regression line for the temperature main effect.

all three seed and seedling variables (df = 1, 152; mean germination rate, $F = 541.19$, $P < 0.0001$; mean rate to 50% coleoptile emergence; $F = 644.69$, $P < 0.0001$; mean rate for coleoptile growth to 2 cm, $F = 298.12$, $P < 0.0001$). Coleoptile mean
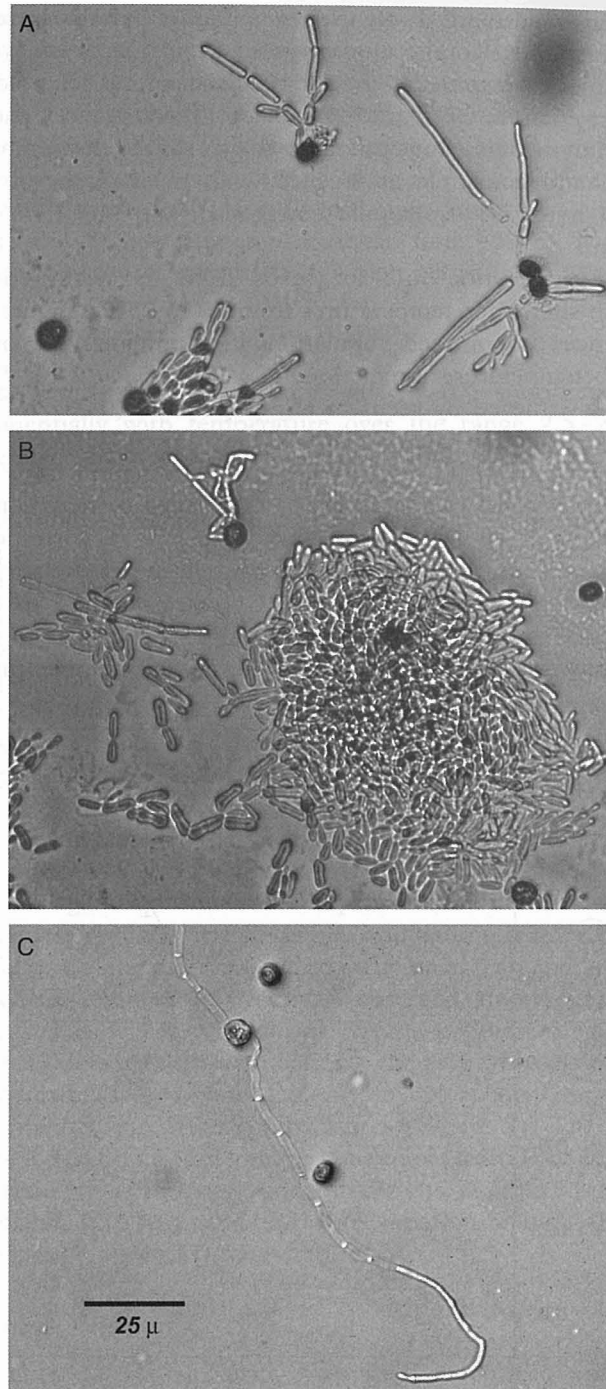
Figure 2. *Ustilago bullata* teliospore germination: (A) after 24 h at 25°C, (B) after 48 h at 25°C, (C) after 984 h at 2.5°C. At 25°C, teliospores germinate by extending a promycelium which then produces numerous haploid sporidia. These mate to produce multiple dikaryotic infection hyphae. At 2.5°C, teliospores germinate directly to the dikaryotic phase, with no extension of a promycelium and no sporidial proliferation. Usually only two dikaryotic hyphae are produced.

emergence rate increased more slowly with temperature than mean seed germination rate, and mean rate of coleoptile elongation to 2 cm increased even more slowly (Figure 3). The times corresponding to maximum mean rates achieved (at 25°C) were 15 h to 50% germination, 27 h to 50% coleoptile emergence, and 71 h to 50% coleoptile elongation to 2 cm. Coleoptile growth was slowed down more at 2.5°C than seed germination and coleoptile emergence, with mean times of 270 h to 50% germination, 345 h to 50% coleoptile emergence, and 909 h to 50% coleoptile elongation to 2 cm.

Average mean germination rate for seeds from the eight co-occurring host populations is also shown for temperatures from 10 to 25°C (Figure 3). There were significant differences among seed populations in germination response to temperature (population × temperature interaction; df = 28, 40; $F = 2.10$; $P = 0.0157$), but the maximum rate achieved by seeds of any population ($0.085 \text{ h}^{-1}$) was still well below teliospore germination rate at 25°C.

At temperatures above 10°C, mean teliospore germination rate (averaged across eight pathogen populations) clearly exceeded mean seed germination rate regardless of seed source, while at 10°C the two values were similar, corresponding to mean germination times of 45 h for seeds and 50 h for teliospores. Relatively slow rates of coleoptile emergence and elongation at 10°C (corresponding to 85 h for mean coleoptile emergence time and 159 h for mean time for elongation to 2 cm) would provide opportunity for infection to take place at this temperature even though
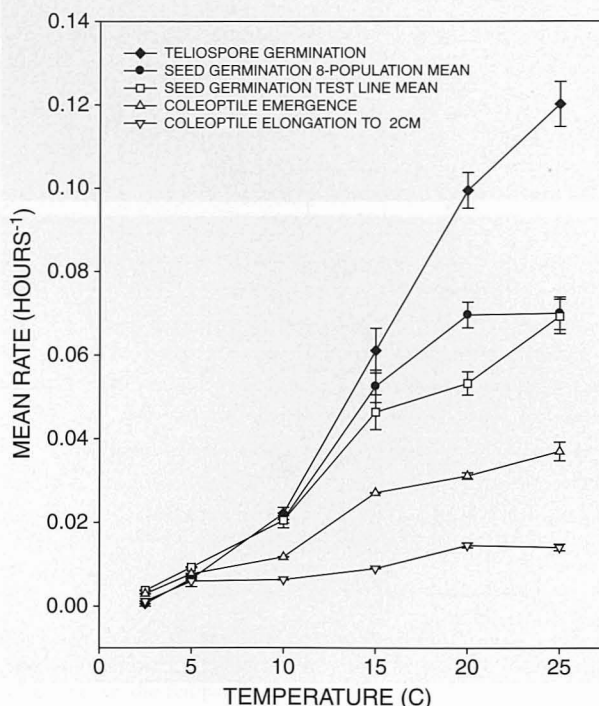


Figure 3. Mean rate as a function of temperature for *Ustilago bullata* teliospore germination (mean of eight pathogen populations) and *Bromus tectorum* seed germination (mean of eight host populations and mean of two Whiterocks test lines), coleoptile emergence (mean of two Whiterocks test lines) and coleoptile elongation to 2 cm (mean of two Whiterocks test lines). Standard error bars are shown.

teliospore germination lags slightly behind. At 5°C, mean seed germination rate clearly exceeded mean teliospore germination rate (mean times of 108 h for seeds, 154 h for teliospores), but mean time for coleoptile elongation to 2 cm was delayed to 169 h, making it at least possible for teliospore germination to catch up before the infection window closes. At 2.5°C, mean seed germination rate greatly exceeded mean teliospore germination rate (mean times of 270 vs. 1754 h). There was little apparent opportunity for infection to take place. Even though coleoptile elongation to 2 cm was delayed considerably at low temperature (mean time 909 h), it is still likely to be completed before teliospores germinate to a high percentage.

### Sporidial proliferation

Mean sporidial proliferation rate (hours to $3 \times 10^7$ sporidial cells per $mL^{-1}$) increased exponentially with temperature over the range 2.5–25°C (temperature main effect for log (mean proliferation rate); $df = 1$, 255; $F = 15538$; $P < 0.0001$; Figure 4).

All populations showed log-linear increase in rate with temperature, but the slope of the relationship varied by population (temperature by population interaction; $df = 7$, 255; $F = 28.54$, $P < 0.0001$; Figure 4). Populations usually had similar proliferation rates at 15°C, but sometimes had widely contrasting rates at higher and lower temperatures. For example, the slope of change with temperature was shallow for the Strawberry population. Its mean proliferation time at 2.5°C was the shortest of any population (523 h), while at 25°C it had the longest mean proliferation time (51 h). The Potosi Pass population had a steeper slope of change with temperature, with the longest mean proliferation time at 2.5°C (1076 h) and one of the shortest times at 25°C (32 h). The Whiterocks population had a slope of change similar to the average slope, but a higher intercept, so that its mean proliferation times at both 2.5 and 25°C were relatively short (601 and 26 h, respectively).

There were also significant differences in proliferation rate among sporidial isolates within a population (isolate nested within population main effect; $df = 32$, 255; $F = 2.22$; $P = 0.0004$). The pattern of more variation at high and low temperature and less at intermediate temperature that was observed among populations was also evident among isolates within populations. Within-population variance, an index of among-isolate variation within a population, was always lowest at the intermediate temperature, indicating less variation among isolates at this temperature, with more variation at the higher and lower temperatures (Figure 5). The magnitude of this effect was also different for different populations, with Potosi Pass showing low among-isolate variation at all three temperatures and Hobble Creek, Strawberry and Whiterocks showing very high among-isolate variation at high (Hobble Creek and Strawberry) or low (Whiterocks) temperatures.

### Growth chamber inoculation trials

In growth chamber experiments using host seeds inoculated with teliospores from eight pathogen populations at two densities and allowed to emerge at a range of temperatures, temperature was the most important variable affecting disease incidence (Table II; Figure 6). Mean disease incidence was highest (90%) at optimum temperature (15 and 20°C). At 25°C mean disease incidence dropped to 72%, while the drop at lower temperatures was more gradual, with a mean of 80% at 10°C and
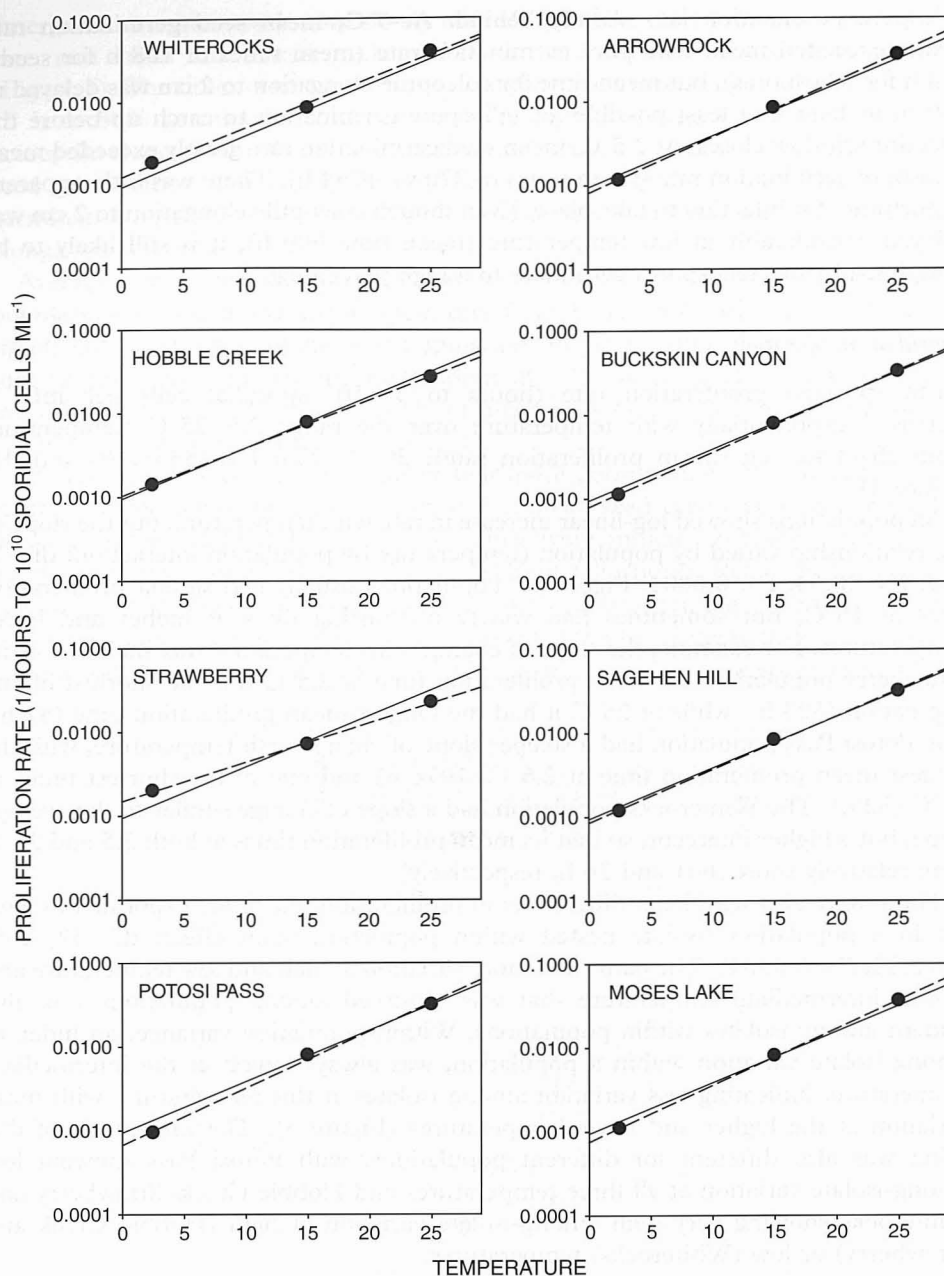
Figure 4. The relationship between log (mean sporidial proliferation rate) and temperature for sporidial isolates from eight populations of *Ustilago bullata*. The dotted lines in each panel represents the population regression line, while the solid line represents the temperature main effect regression line.

65% at 5°C. Mean disease incidence at 2.5°C was less than 5%. Disease incidence levels were significantly lower at the lower inoculum density (Table II; Figure 6). The impact of inoculum density on disease incidence increased with distance from the optimum temperature, as evidenced in the significant inoculum density by
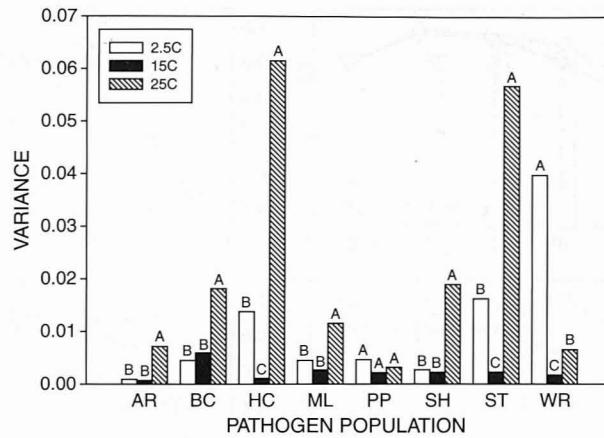
Figure 5. Within-population variance in log (mean sporidial proliferation rate), an index of variation among isolates within populations, at three temperatures for sporidial isolates from each of eight populations of *Ustilago bullata*. Within a population, variances headed by the same letter are not significantly different ($P < 0.05$) according to Levy's multiple comparison test for differences among variances (AR =Arrowrock, BC =Buckskin Canyon, HC =Hobble Creek, ML =Moses Lake, PP = Potosi Pass, SH =Sagehen Hill, ST =Strawberry, WR =Whiterocks).

temperature interaction. The exception was at 2.5°C, where disease incidence was generally very low regardless of inoculum density.

There were marginally significant differences among pathogen populations in the ability to cause disease as a function of temperature (Table II; Figure 6). These were generally manifested as differential responses to super- and sub-optimal temperatures. For example, the Hobble Creek, and Potosi Pass populations showed no decrease in disease incidence at 25°C relative to the optimum, while the Arrowrock, Buckskin Canyon, Sagehen Hill and Moses Lake populations showed sharp decreases.

*Field inoculation trial*

Mean disease incidence in the early field planting was 84%, while the late planting showed <2% disease incidence, a highly significant difference (df = 1, 20.7,

Table II. Statistical analysis of the growth chamber inoculation experiment using the SAS mixed model procedure for a split plot design, with time repetition and temperature as main plot factors, block nested within time repetition and temperature, and pathogen population and inoculum density as subplot factors. Repetition in time and block were treated as random effects, while temperature, inoculum density, and pathogen population were treated as fixed effects.

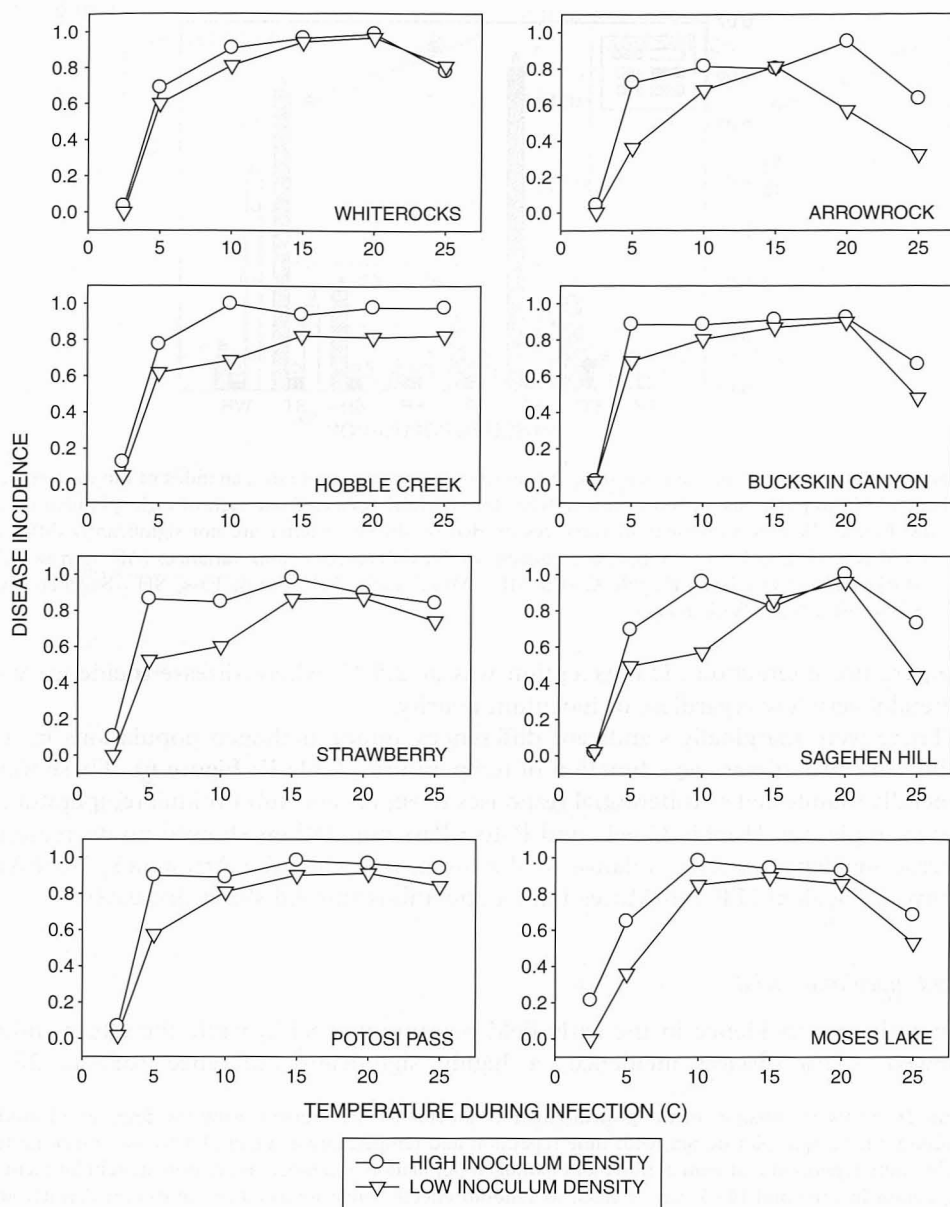| Effect | Effect df | Error df | $F$ value | $P$ value |
|---|---|---|---|---|
| Temperature | 6 | 7 | 17.79 | 0.0006 |
| Population | 7 | 160 | 5.88 | <0.0001 |
| Density | 1 | 29.1 | 105.51 | <0.0001 |
| Population by temperature | 42 | 160 | 1.40 | 0.0740 |
| Population by density | 7 | 138 | 1.92 | 0.0707 |
| Temperature by density | 6 | 29.1 | 5.68 | 0.0341 |
| Pop. × temperature × density | 42 | 138 | 1.04 | 0.4188 |

Figure 6. Disease incidence on *Bromus tectorum* as a function of pathogen population, temperature, and inoculum density in growth chamber inoculation experiments with teliospore collections from eight populations of *Ustilago bullata*.

$F = 1796.5$, $P < 0.0001$; Figure 7). None of the other main effects or interactions were significant, although the population main effect was marginally so (df $= 7$, 227; $F = 1.74$; $p = 0.100$). Further analysis showed that differences among pathogen populations were significant for the early planting date (df $= 7$, 222, $F = 3.13$, $p = 0.0036$; Figure 7) but not for the late planting date. Potosi Pass, the warm desert
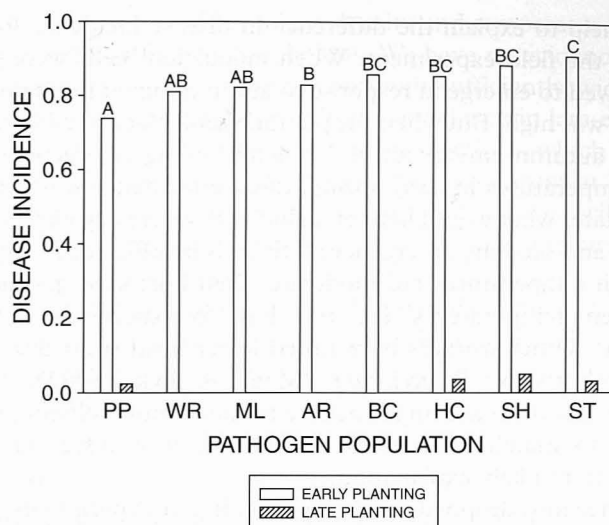
Figure 7. Disease incidence on *Bromus tectorum* as a function of pathogen population and planting date in a field inoculation experiment with teliospore collections from eight populations of *Ustilago bullata* (Table I). Columns for the early planting date headed by the same letter are not significantly different at the $P=0.05$ level according to means separations from mixed model analysis of variance (AR = Arrowrock, BC = Buckskin Canyon, HC = Hobble Creek, ML = Moses Lake, PP = Potosi Pass, SH = Sagehen Hill, ST = Strawberry, WR = Whiterocks).

population, produced the lowest disease incidence from the early planting date (74%), while Strawberry, the high montane population, produced the highest (91%).

## Discussion

Our experiments clearly showed that head smut disease incidence on *B. tectorum* is strongly affected by temperature over the range of temperatures likely to be encountered by this pathogen during host seed germination and seedling emergence, when susceptibility to infection is high. At temperatures characteristic of early autumn seed beds (ca. 15–20°C), *U. bullata* teliospore germination proceeds at rates exceeding rates of seed germination. Sporidial proliferation at these temperatures is also able to keep pace with seedling development, making infection even more likely. Disease incidence was high (mean 90%) when inoculated seeds were allowed to emerge at these temperatures. At late autumn seed bed temperatures (ca. 5–10°C), teliospore germination rates lag behind seed germination rates, but slow seedling development rates at these temperatures combined with relatively fast sporidial proliferation rates makes some infection possible, especially at higher inoculum densities. At winter seed bed temperatures (2.5°C), teliospore germination lags far behind seed germination, and even though seedling development rates are slow, teliospore germination cannot not keep pace. Sporidial proliferation does occur at 2.5°C, at a rate more or less commensurate with seedling development. But telio-spore germination at 2.5°C did not result in sporidial proliferation, because germination proceeded directly to the dikaryotic stage. Disease incidence for seedlings emerging at 2.5°C was uniformly low, probably because of very slow teliospore germination.

These results help to explain the difference in disease incidence for early and late planting dates in the field experiment. When inoculated seeds were planted in early October and allowed to emerge in response to autumn rain at moderate temperatures, disease incidence was high. But when the planting took place in early winter, obviating the possibility of autumn emergence and instead forcing emergence to take place at relatively cold temperatures in early spring, disease incidence was very low. The late planting date imitates what would happen in habitats where dry weather limits autumn host germination and seedling emergence. If there is insufficient precipitation for seed germination when temperatures are moderate, then host seed germination will take place later, when temperatures are too low for successful infection (Meyer unpublished data). Other workers have noted lower head smut disease incidence in later-emerging cohorts of *B. tectorum* (Mack & Pyke 1983). Hulbert (1955) hypothesized that this decrease might be due to inoculum depletion, but the inability of the pathogen to match its development to the host infection window at low temperature is a more likely explanation.

Disease incidence also dropped off above 20°C in our experiments, which indicates that 25°C is super-optimal for infection. In Falloon's work with *B. catharticus*, 25°C was the optimum temperature for infection, with decreases in disease incidence only at even higher temperature (Falloon 1979a). *Ustilago bullata* populations on *B. tectorum* may be specifically adapted to operate at moderate autumn seed bed temperatures, as suggested by Turnbull and Gossen (2000). In our data set, both teliospore germination and sporidial proliferation rates increased linearly to 25°C, indicating that, for these processes, 25°C is at or below the optimum temperature. This indicates that some other stage of the infection process, such as mating, dikaryon formation, or host penetration, is limited at 25°C relative to lower temperatures, resulting in lower infection. There would be little selection on the pathogen to show an adaptive response at this temperature. Host seeds rarely germinate in the field at temperatures as high as 25°C, because during the summer, when such temperatures are likely to coincide with precipitation and moist seed beds, the seeds are dormant (Bauer et al. 1998).

We measured teliospore germination and sporidial proliferation on PDA, a high-nutrient environment similar to that probably found on the surface of the seed over the embryo. Falloon (1979a) noted that spore germination and sporidial proliferation proceeded much more quickly on the thin membrane directly over the embryo of *B. catharticus* seeds than on the lemma, which is much thicker and further from the embryo. Similarly, we found that 24-h teliospore germination on PDA was much higher than on water agar over a range of temperatures (Boguena 2003).

We saw an effect of inoculum density in our growth chamber experiment but not in our field experiment. We used higher inoculum densities in the field experiment with the idea that higher densities might be needed to obtain maximum infection under suboptimal field conditions, but this was not the case. We obtained high levels of infection in the field regardless of inoculum density, probably because even at the lower density, inoculum load was no longer the main factor limiting infection. Our goal in these experiments was to maximize infection in the context of biocontrol, not to attempt to simulate natural inoculum loads. We know little about naturally occurring inoculum loads, primarily because of measurement difficulties.

We found significant among-population differences in both teliospore germination rate and sporidial proliferation rate as a function of temperature. But the pattern of

disease incidence as a function of inoculum density and temperature during infection was quite similar among pathogen populations. We have no evidence from this study that it would be possible to find a population with sufficiently wide environmental tolerance to overcome the problem of low disease incidence in late-emerging seedling cohorts of the host. None of the tested pathogen populations, which run the gamut of habitats occupied by the host, were able to infect to any extent at low temperature. Effective use of this pathogen as a biocontrol agent for *B. tectorum* will likely be limited to environments with a consistent pattern of early autumn precipitation, where host germination takes place under moderately warm conditions conducive to infection. Results from our field trial suggest that it may be possible to produce major head smut epidemics through artificial inoculation under this scenario. Further studies will investigate the practicality of this approach for *B. tectorum* biocontrol in both croplands and wildlands.

## Acknowledgements

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